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(54) HEK293 CELL LINE FOR ACTIVITY STUDIES AND HTS SYSTEM OF ALPHA1G T-TYPE CA2+ CHANNEL

(76) Inventors: **Ye Sun Han**, Seoul (KR); **Hyewhon Rhim**, Seoul (KR); **Taehyun Kim**, Seoul (KR); **Sunoh Kim**, Seoul (KR); **Dae-sik Lim**, Daejeon (KR); **Juhyun Choi**, Bucheon-si (KR)

Correspondence Address:
JONES DAY
222 EAST 41ST ST
NEW YORK, NY 10017 (US)

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(57) **ABSTRACT**

There is provided a cell line that is prepared by transforming HEK293 cell with a human Kir2.1 gene using a retrovirus expression system, wherein the HEK293 expresses a stable α_{1G} T-type calcium channel. The cell line responds sensitively to KCl and forms an appropriate level of the membrane voltage so that the cell signaling pathway may be investigated by the molecular biological and biochemical studies.

```
1    ATGGGCAGTGTGCGAACCAACCGCTACAGCATCGTCTCTTCAGAAGAAGACGGTATGAAG 60
       M  G  S  V  R  T  N  R  Y  S  I  V  S  S  E  E  D  G  M  K
61   TTGGCCACCATGGCAGTTGCAAATGGCTTTGGGAACGGGAAGAGTAAAGTCCACACCCGA 120
       L  A  T  M  A  V  A  N  G  F  G  N  G  K  S  K  V  H  T  R
121  CAACAGTGCAGGAGCCGCTTTGTGAAGAAAGATGGCCACTGTAATGTTCAGTTCATCAAT 180
       Q  Q  C  R  S  R  F  V  K  K  D  G  H  C  N  V  Q  F  I  N
181  GTGGGTGAGAAGGGGCAACGGTACCTCGCAGACATCTTCACCACGTGTGTGGACATTCGC 240
       V  G  E  K  G  Q  R  Y  L  A  D  I  F  T  T  C  V  D  I  R
241  TGGCGGTGGATGCTGGTTATCTTCTGCCTGGCTTTCGTCCTGTCATGGCTGTTTTTTGGC 300
       W  R  W  M  L  V  I  F  C  L  A  F  V  L  S  W  L  F  F  G
301  TGTGTGTTTTGGTTGATAGCTCTGCTCCATGGGGACCTGGATGCATCCAAAGAGGGCAAA 360
       C  V  F  W  L  I  A  L  L  H  G  D  L  D  A  S  K  E  G  K
361  GCTTGTGTGTCCGAGGTCAACAGCTTCACGGCTGCCTTCCTCTTCTCCATTGAGACCCAG 420
       A  C  V  S  E  V  N  S  F  T  A  A  F  L  F  S  I  E  T  Q
421  ACAACCATAGGCTATGGTTTCAGATGTGTCACGGATGAATGCCCAATTGCTGTTTTCATG 480
       T  T  I  G  Y  G  F  R  C  V  T  D  E  C  P  I  A  V  F  M
481  GTGGTGTTCCAGTCAATCGTGGGCTGCATCATCGATGCTTTCATCATTGGCGCAGTCATG 540
       V  V  F  Q  S  I  V  G  C  I  I  D  A  F  I  I  G  A  V  M
541  GCCAAGATGGCAAAGCCAAAGAAGAGAAACGAGACTCTTGTCTTCAGTCACAATGCCGTG 600
       A  K  M  A  K  P  K  K  R  N  E  T  L  V  F  S  H  N  A  V
601  ATTGCCATGAGAGACGGCAAGCTGTGTTTGATGTGGCGAGTGGGCAATCTTCGGAAAAGC 660
       I  A  M  R  D  G  K  L  C  L  M  W  R  V  G  N  L  R  K  S
661  CACTTGGTGGAAGCTCATGTTCGAGCACAGCTCCTCAAATCCAGAATTACTTCTGAAGGG 720
       H  L  V  E  A  H  V  R  A  Q  L  L  K  S  R  I  T  S  E  G
721  GAGTATATCCCTCTGGATCAAATAGACATCAATGTTGGGTTTGACAGTGGAATCGATCGT 780
       E  Y  I  P  L  D  Q  I  D  I  N  V  G  F  D  S  G  I  D  R
781  ATATTTCTGGTGTCCCCAATCACTATAGTCCATGAAATAGATGAAGACAGTCCTTTATAT 840
       I  F  L  V  S  P  I  T  I  V  H  E  I  D  E  D  S  P  L  Y
841  GATTTGAGTAAACAGGACATTGACAACGCAGACTTTGAAATCGTGGTCATACTGGAAGGC 900
       D  L  S  K  Q  D  I  D  N  A  D  F  E  I  V  V  I  L  E  G
901  ATGGTGGAAGCCACTGCCATGACGACACAGTGCCGTAGCTCTTATCTAGCAAATGAAATC 960
       M  V  E  A  T  A  M  T  T  Q  C  R  S  S  Y  L  A  N  E  I
961  CTGTGGGGCCACCGCTATGAGCCTGTGCTCTTTGAAGAGAAGCACTACTACAAAGTGGAC 1020
       L  W  G  H  R  Y  E  P  V  L  F  E  E  K  H  Y  Y  K  V  D
```

FIG. 1a

```
1021 TATTCCAGGTTCCACAAAACTTACGAAGTCCCCAACACTCCCCTTTGTAGTGCCAGAGAC 1080
      Y  S  R  F  H  K  T  Y  E  V  P  N  T  P  L  C  S  A  R  D
1081 TTAGCAGAAAAGAAATATATCCTCTCAAATGCAAATTCATTTTGCTATGAAAATGAAGTT 1140
      L  A  E  K  K  Y  I  L  S  N  A  N  S  F  C  Y  E  N  E  V
1141 GCCCTCACAAGCAAAGAGGAAGACGACAGTGAAAATGGAGTTCCAGAAAGCACTAGTACG 1200
      A  L  T  S  K  E  E  D  D  S  E  N  G  V  P  E  S  T  S  T
1201 GACACGCCCCCTGACATAGACCTTCACAACCAGGCAAGTGTACCTCTAGAGCCCAGGCCC 1260
      D  T  P  P  D  I  D  L  H  N  Q  A  S  V  P  L  E  P  R  P
1261 TTACGGCGAGAGTCGGAGATA 1281
      L  R  R  E  S  E  I
```

FIG. 1b

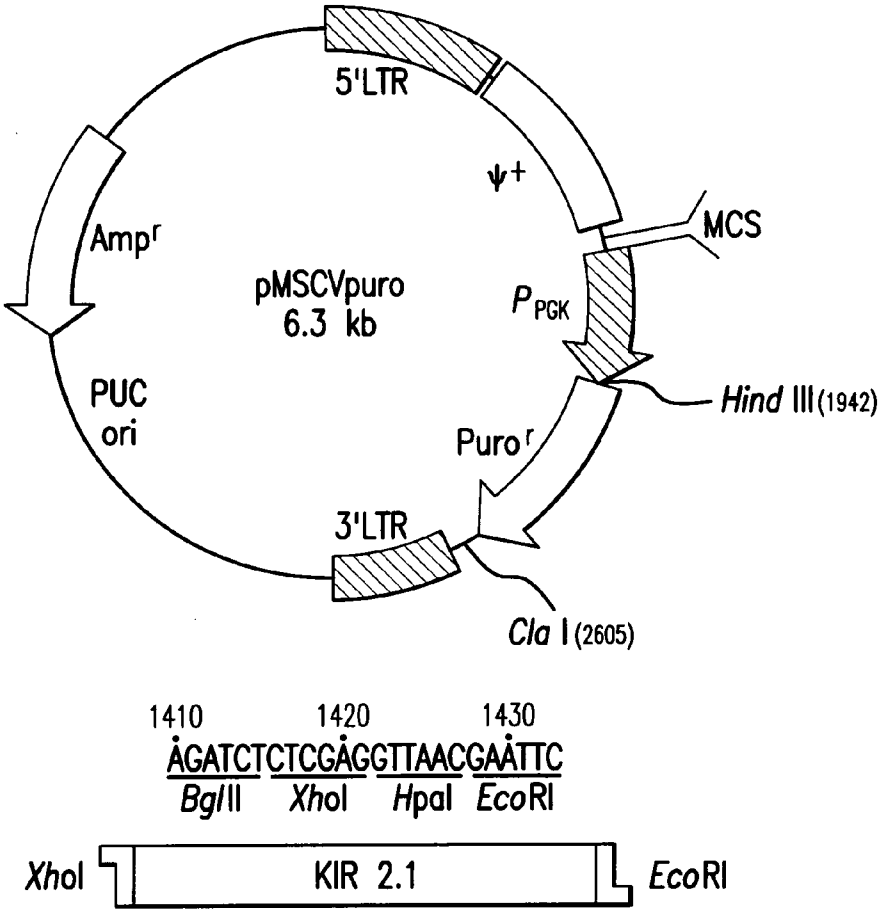
5'LTR
ψ+
MCS
Amp^r
pMSCVpuro
6.3 kb
P PGK
PUC
ori
Hind III (1942)
Puro^r
3'LTR
Cla I (2605)
1410
1420
1430
AGATCTCTCGAGGTTAACGAATTC
BglII
XhoI
HpaI
EcoRI
XhoI
KIR 2.1
EcoRI

FIG.2

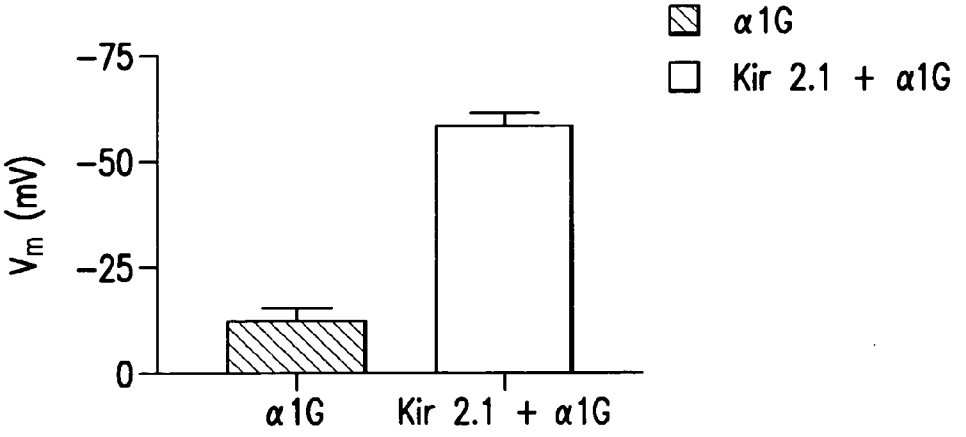
α1G
Kir 2.1 + α1G
−75
−50
−25
0
Vm (mV)
α1G
Kir 2.1 + α1G

FIG.3

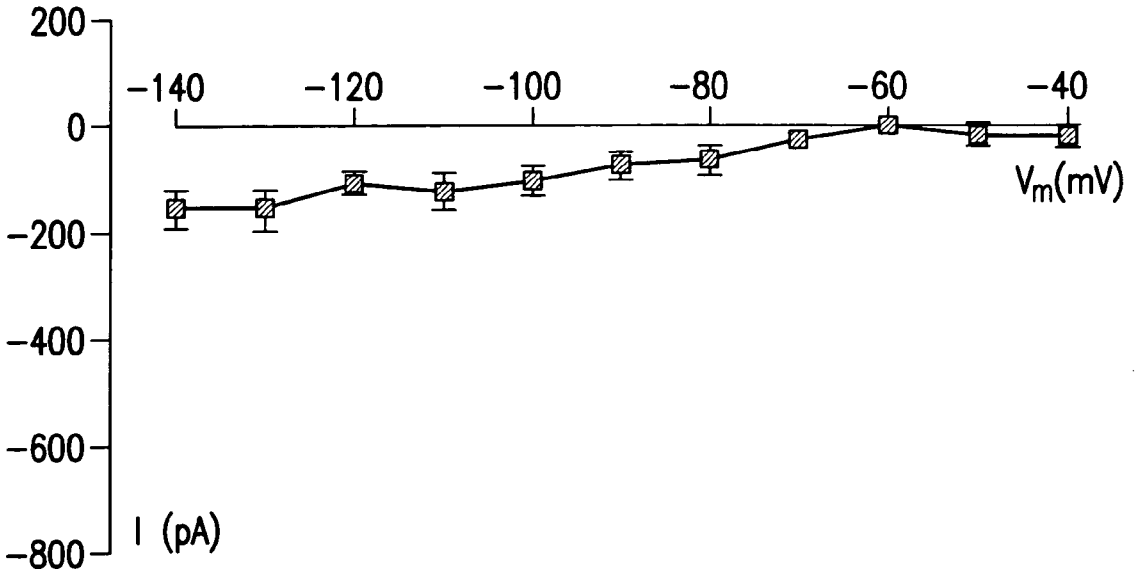

FIG.4a

Kir 2.1 + α1G

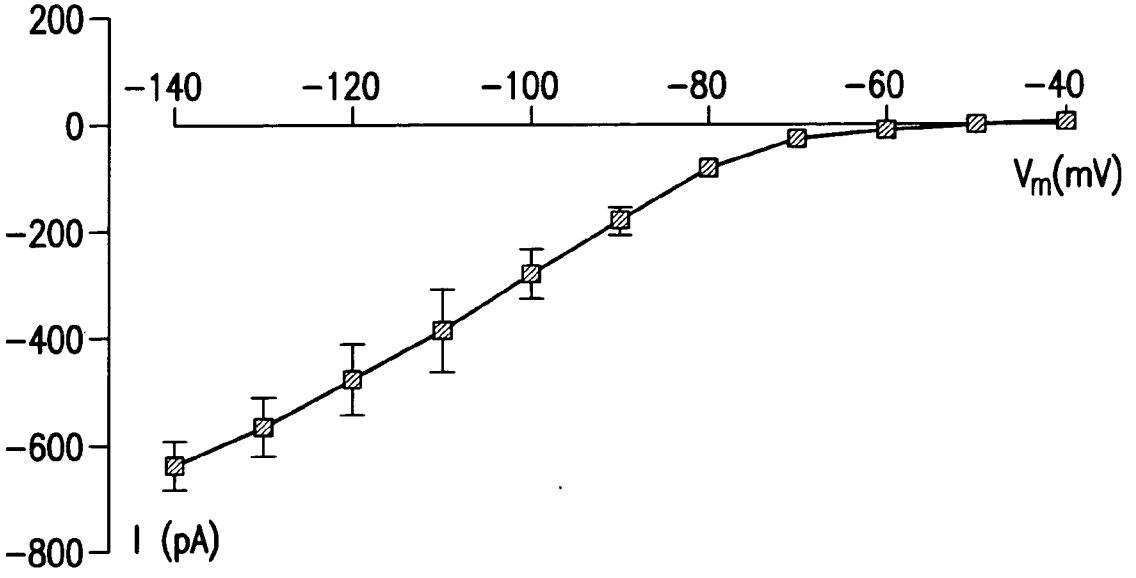

FIG.4b

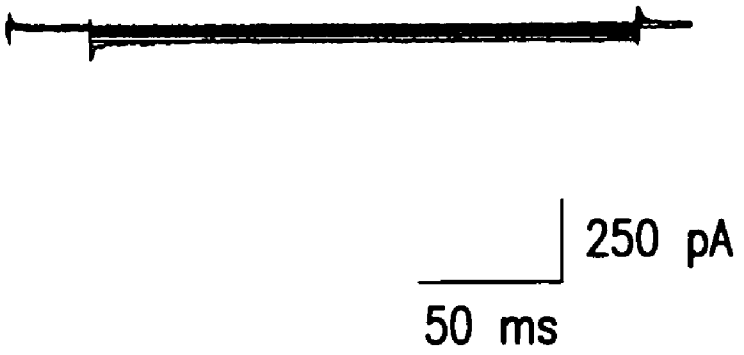

FIG.4c

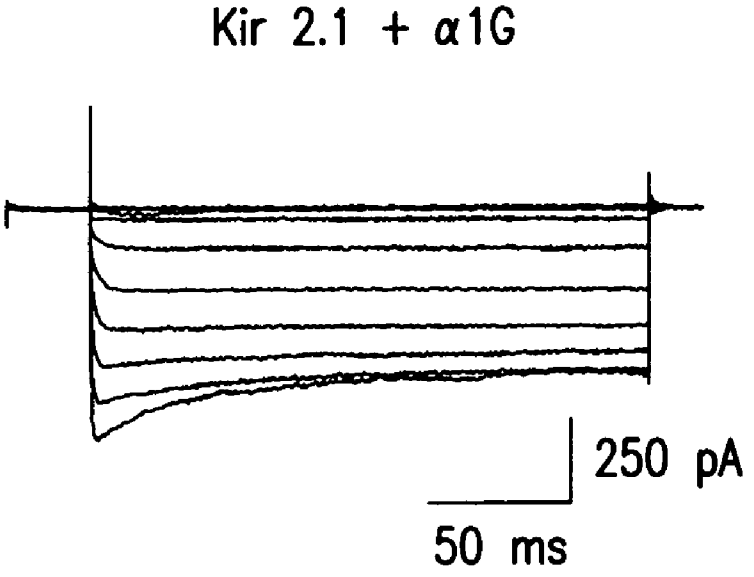

FIG.4d

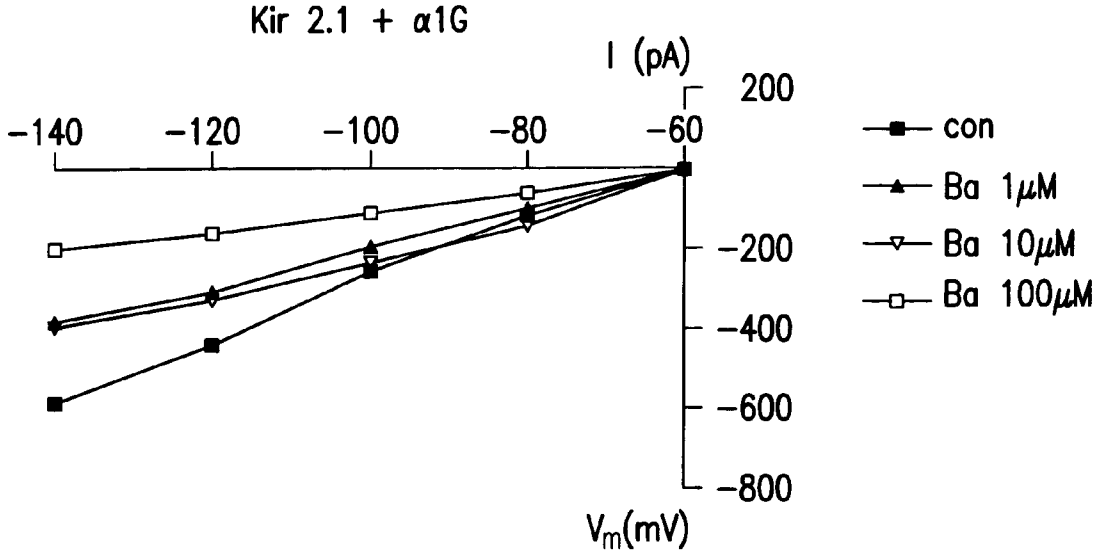
Kir 2.1 + α1G
I (pA)
200
-140
-120
-100
-80
-60
-200
-400
-600
-800
Vm(mV)
con
Ba 1μM
Ba 10μM
Ba 100μM

FIG.4e

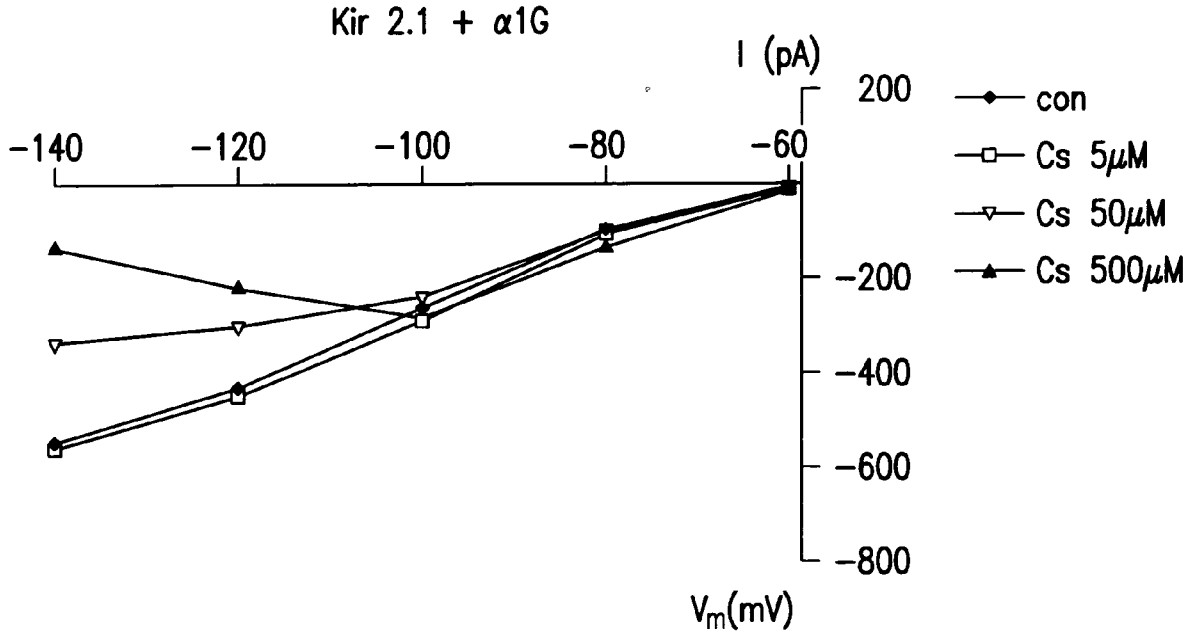
Kir 2.1 + α1G
I (pA)
200
-140
-120
-100
-80
-60
-200
-400
-600
-800
Vm(mV)
con
Cs 5μM
Cs 50μM
Cs 500μM

FIG.4f

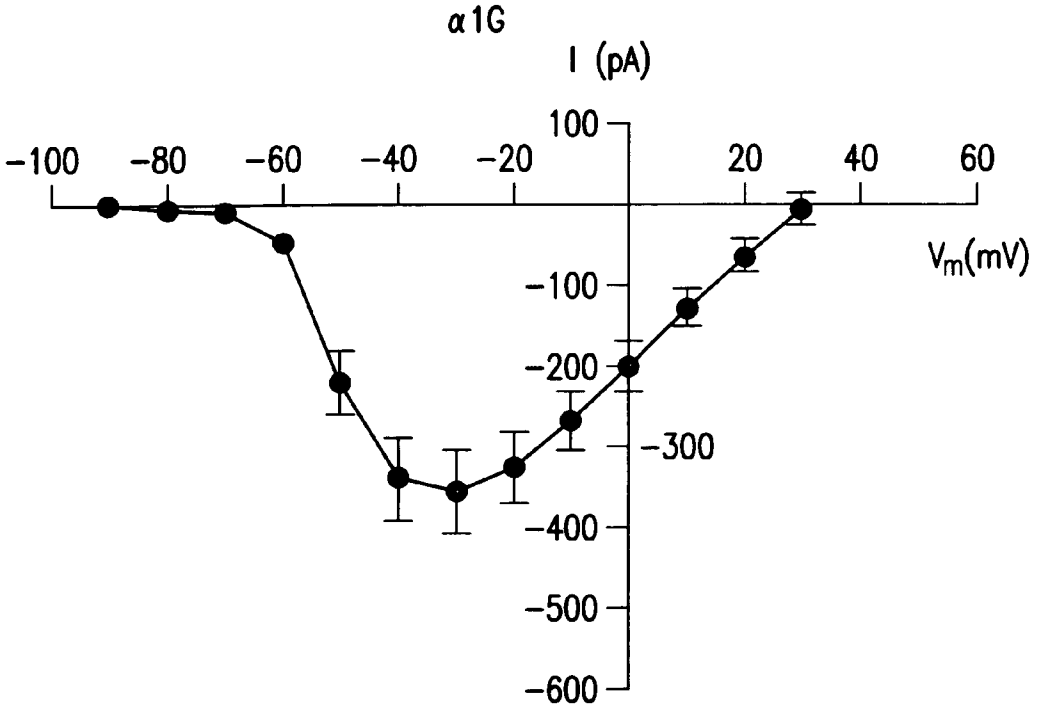
α1G
I (pA)
100
-100
-80
-60
-40
-20
20
40
60
Vm(mV)
-100
-200
-300
-400
-500
-600

FIG.5a

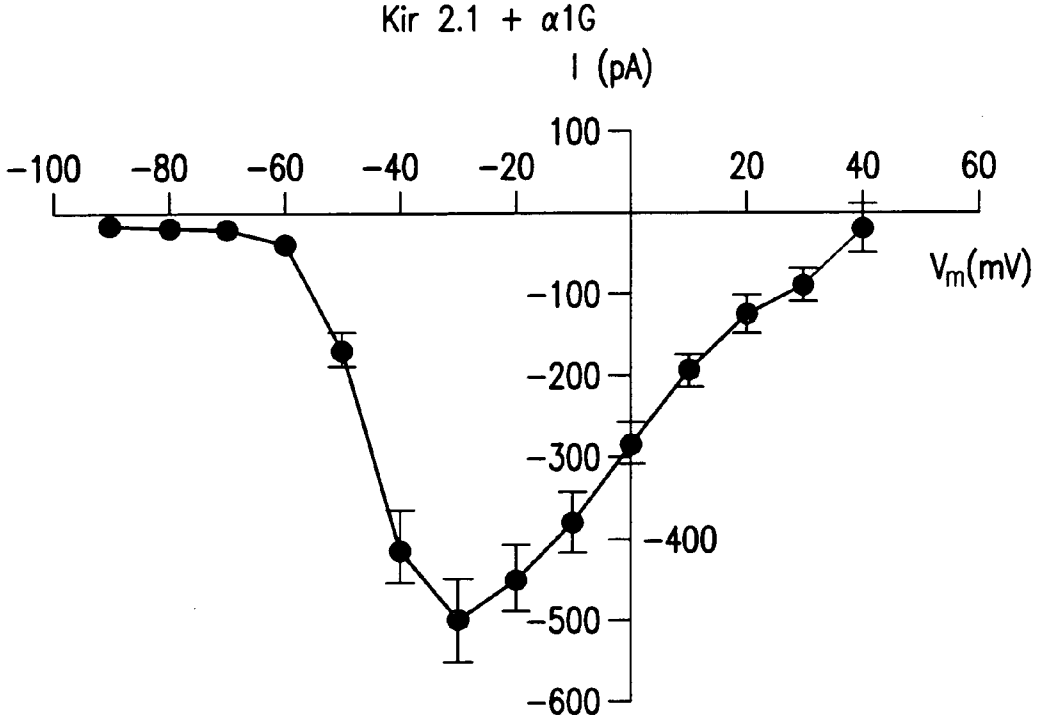
Kir 2.1 + α1G
I (pA)
100
-100
-80
-60
-40
-20
20
40
60
Vm(mV)
-100
-200
-300
-400
-500
-600

FIG.5b

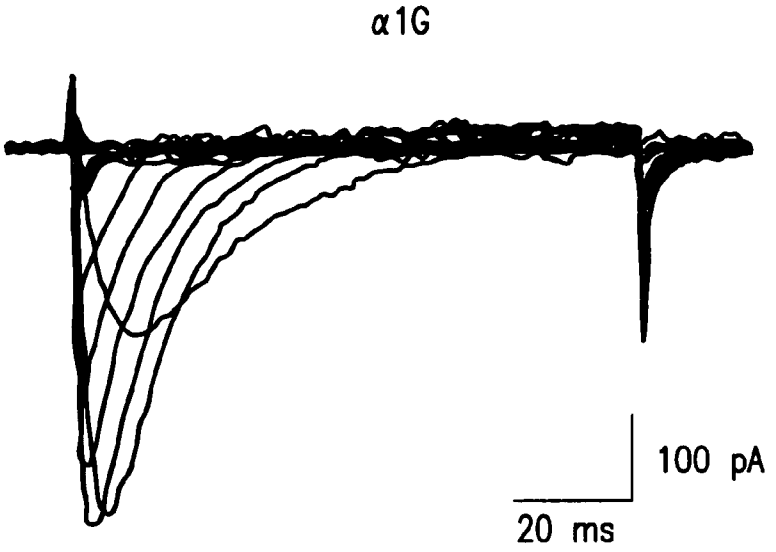

FIG.5c

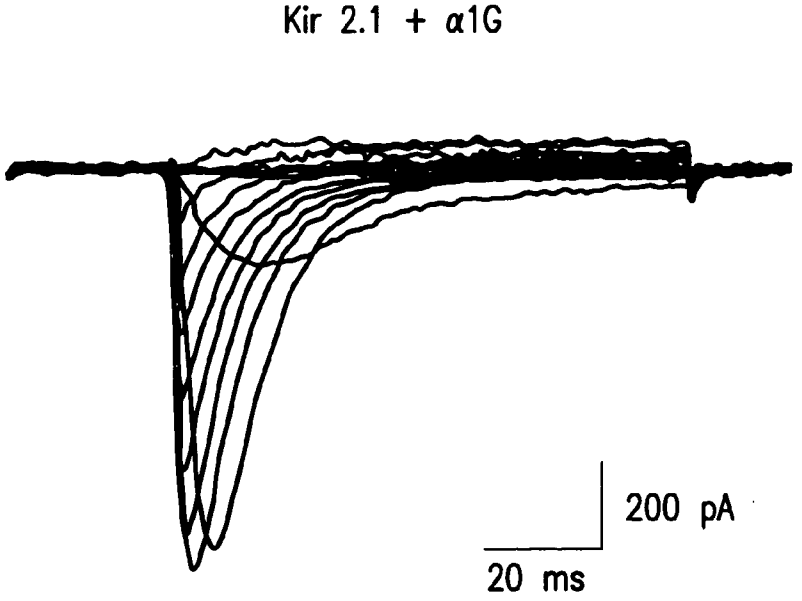

FIG.5d

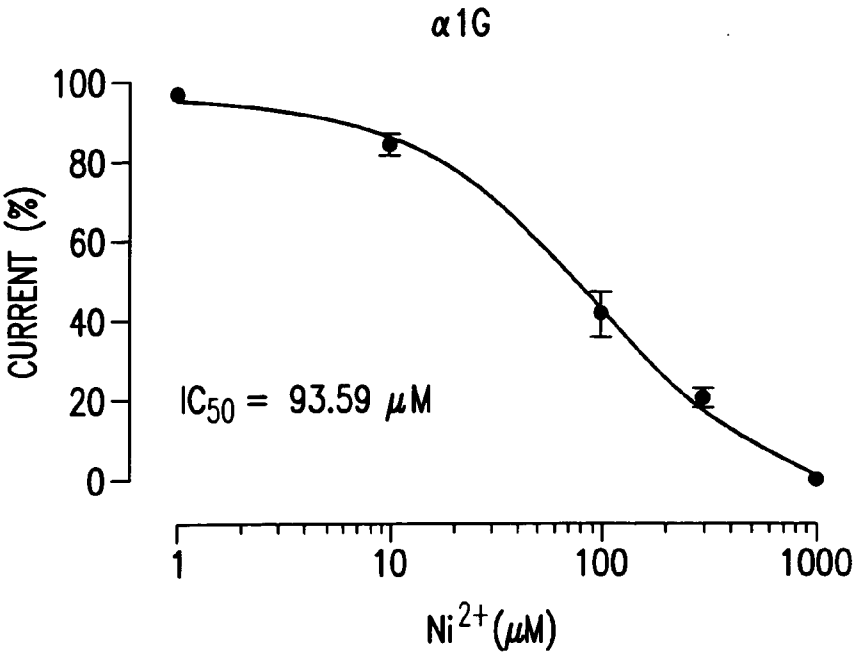
α1G
100
80
60
40
20
0
CURRENT (%)
IC50 = 93.59 μM
1
10
100
1000
Ni2+(μM)

FIG.5e

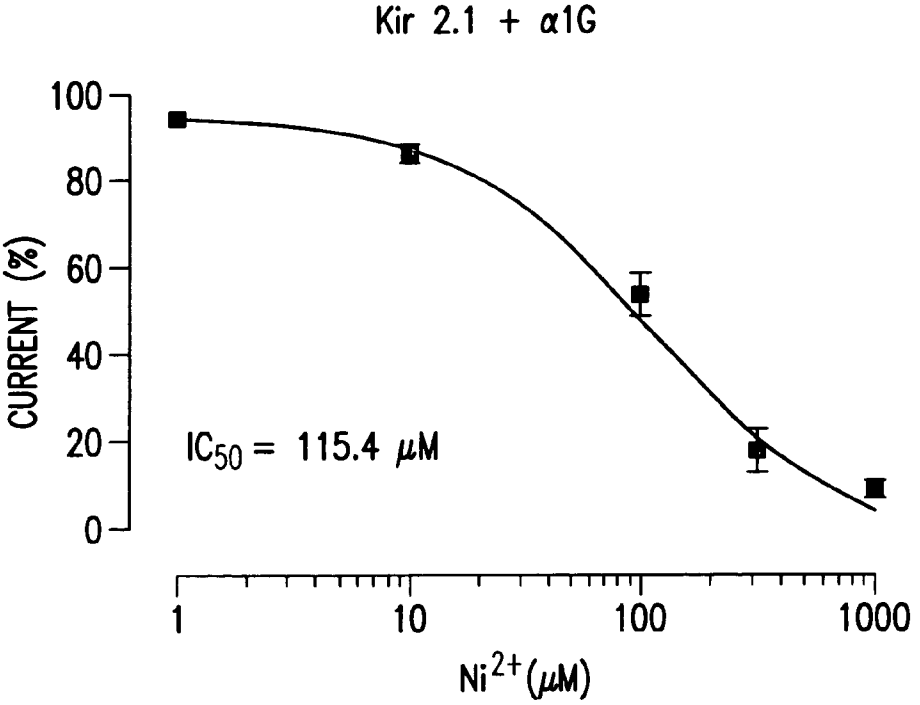
Kir 2.1 + α1G
100
80
60
40
20
0
CURRENT (%)
IC50 = 115.4 μM
1
10
100
1000
Ni2+(μM)

FIG.5f

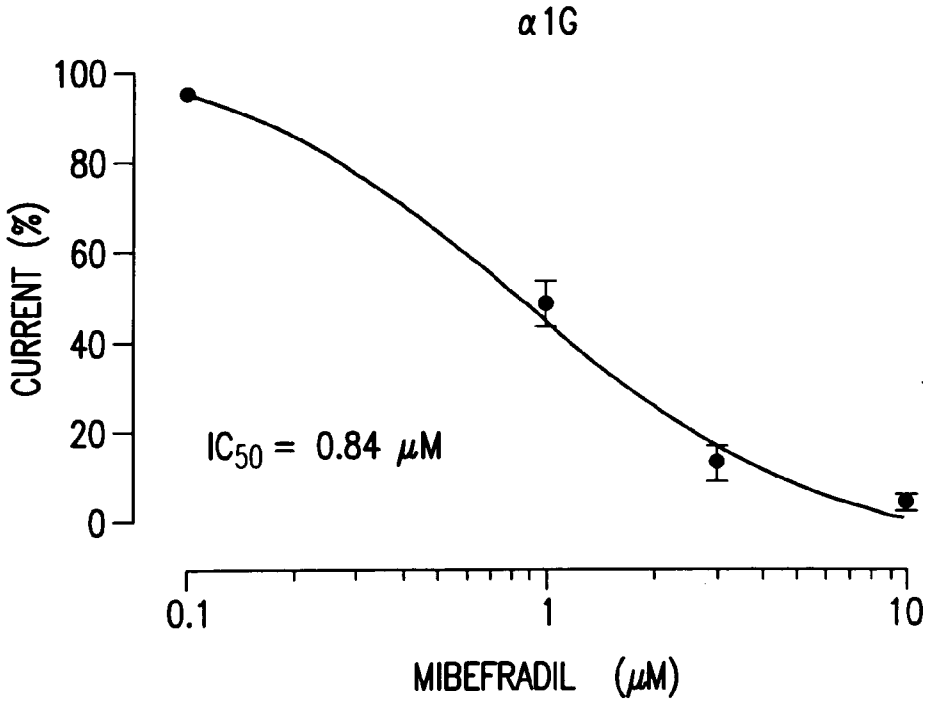
α1G
100
80
60
40
20
0
CURRENT (%)
IC50 = 0.84 μM
0.1
1
10
MIBEFRADIL (μM)

FIG.5g

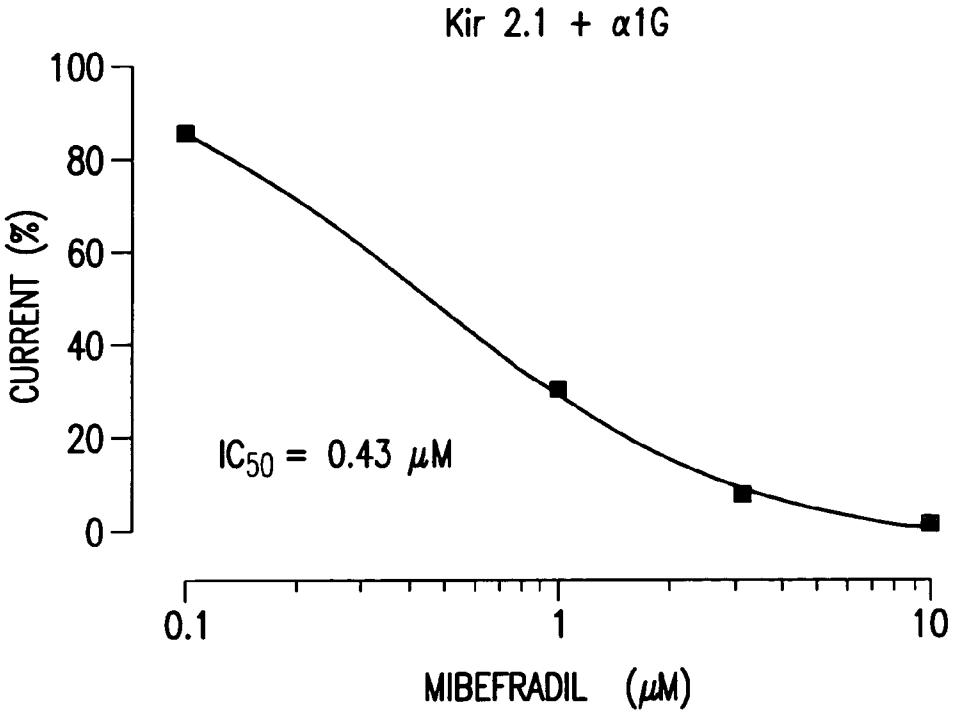
Kir 2.1 + α1G
100
80
60
40
20
0
CURRENT (%)
IC50 = 0.43 μM
0.1
1
10
MIBEFRADIL (μM)

FIG.5h

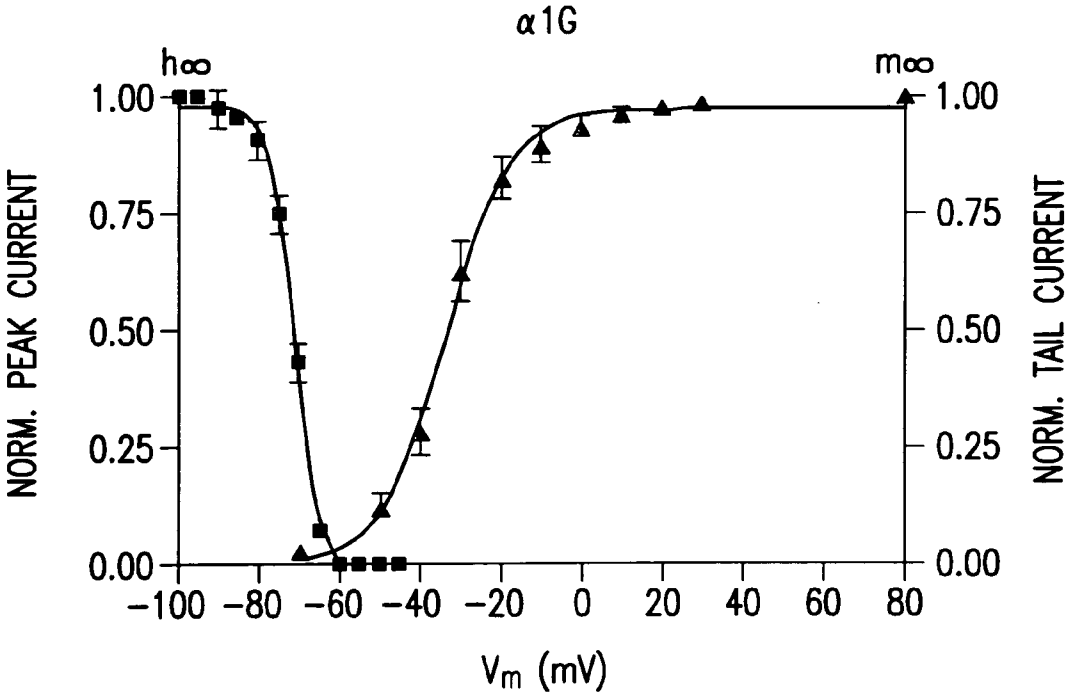
α1G
hoo
moo
1.00
0.75
0.50
0.25
0.00
NORM. PEAK CURRENT
NORM. TAIL CURRENT
-100 -80 -60 -40 -20 0 20 40 60 80
Vm (mV)

FIG.6a

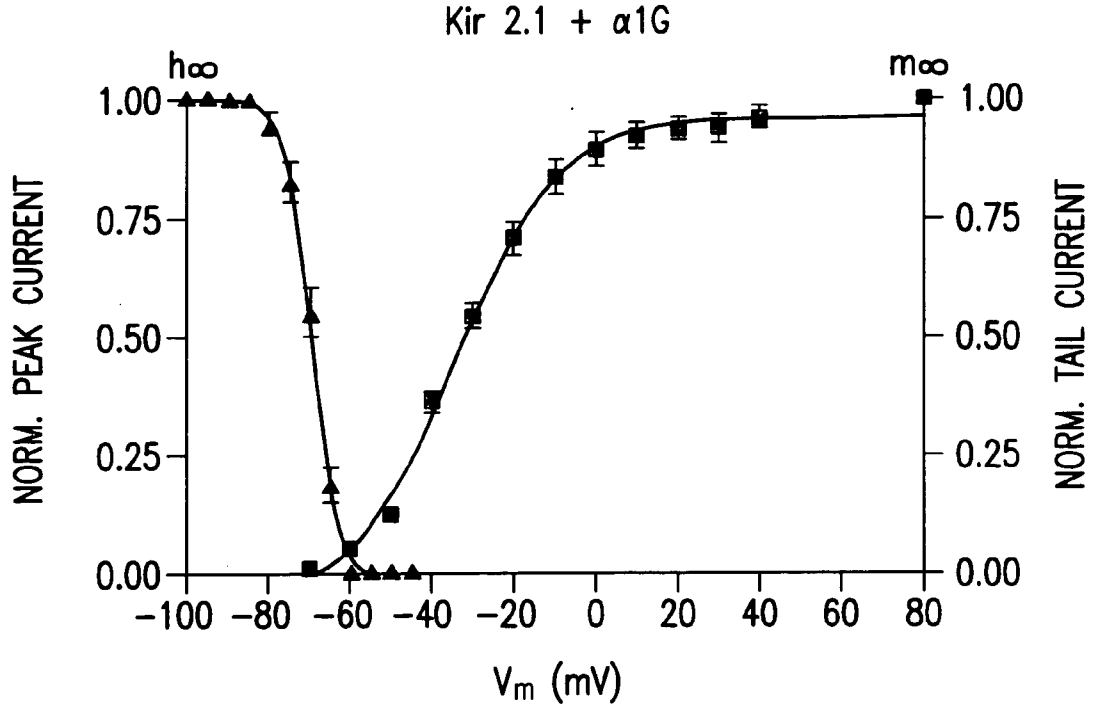
Kir 2.1 + α1G
hoo
moo
1.00
0.75
0.50
0.25
0.00
NORM. PEAK CURRENT
NORM. TAIL CURRENT
-100 -80 -60 -40 -20 0 20 40 60 80
Vm (mV)

FIG.6b

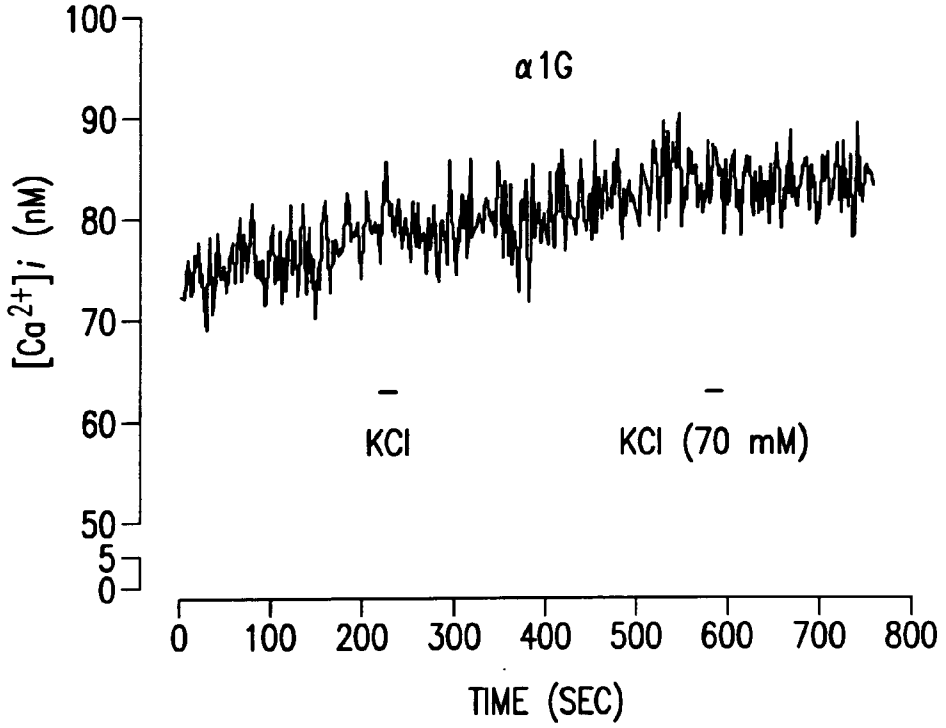
α1G
100
90
80
70
60
50
5
0
[Ca2+]i (nM)
KCl
KCl (70 mM)
0
100
200
300
400
500
600
700
800
TIME (SEC)

FIG.7a

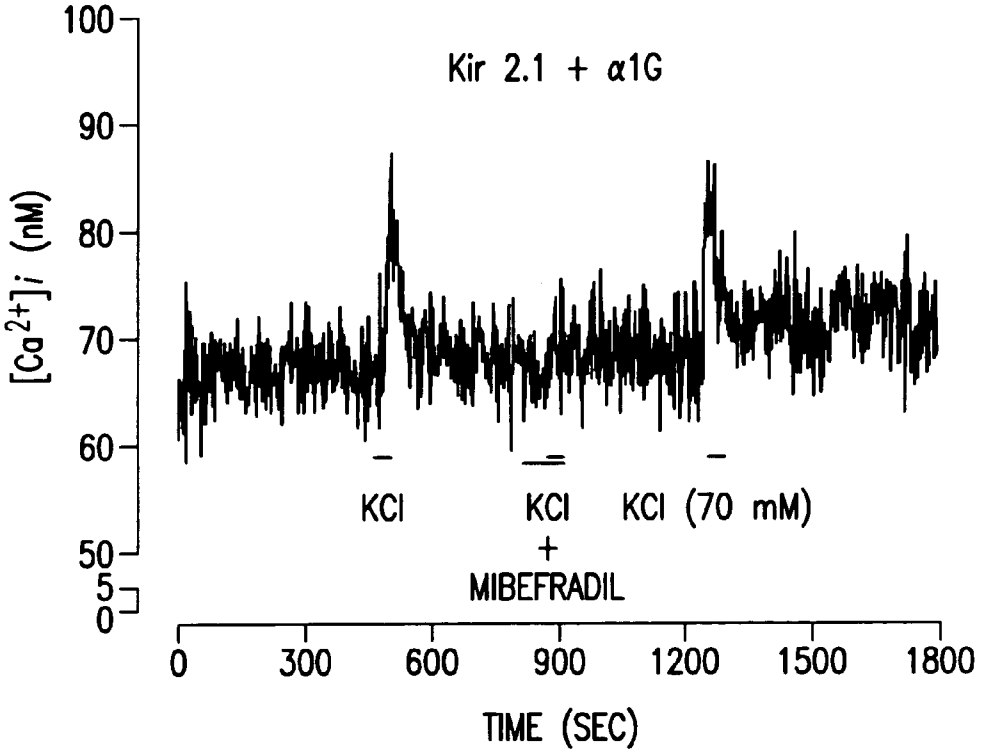
Kir 2.1 + α1G
100
90
80
70
60
50
5
0
[Ca2+]i (nM)
KCl
KCl
+
MIBEFRADIL
KCl (70 mM)
0
300
600
900
1200
1500
1800
TIME (SEC)

FIG.7b

HEK293 CELL LINE FOR ACTIVITY STUDIES AND HTS SYSTEM OF ALPHA1G T-TYPE CA2+ CHANNEL

FIELD OF THE INVENTION

[0001] The present invention relates generally to cell lines, and more particularly to a cell line that is prepared by transforming certain HEK293 cell with a human Kir2.1 gene using a retrovirus expression system.

BACKGROUND OF THE INVENTION

[0002] A membrane protein, voltage-dependent Ca^{2+} channel controls various intracellular actions such as muscular contraction, nerve cell generation and synaptic plasticity, secretion of neutrotransmitter and hormone, and gene expression by regulating calcium ion influx from outside of a cell. As is well known in the art, such voltage-dependent calcium channel is divided into the two main groups based on the requirement voltage for opening channels: high-voltage-activated (HVA) and low-voltage-activated (LVA) Ca^{2+} channels. Currently, HVA Ca^{2+} channels are further divided into L-, N-, P/Q-, and R-types and these functional diversities are related to the existence of several α_1 subunits ($\alpha_{1A\text{-}F}$ and α_{1S}). LVA Ca^{2+} channels are readily distinguished from HVA Ca^{2+} channels because they activate at potential near the resting membrane potential and referred to as "transient (T)-type Ca^{2+} channels" due to their fast inactivation and small conductance. Until recently, three genes encoding T-type Ca^{2+} channel pore-forming subunits were identified and designated $Ca_v3.1$ (α_{1G}), $Ca_v3.2$ (α_{1H}), and $Ca_v3.3$ (α_{1I}).

[0003] Among the above-noted calcium channels, the T-type calcium channel is known to have many functional aspects which are well defined in various printed publications. The functions of T-type calcium channel include and extend to, for instance, controlling the firing bursts of a nerve cell (See, Huguenard, J. R. et al., Annu. Rev. Physiol. 58, 329-348, 1996), pace maker activity of the heart (See, Zhou, Z & Lipsius, S. L. J. Mol. Cell. Cardiol. 26, 1211-1219, 1994), a hormone aldosterone secretion (See, Rossier, M. F. et al., Endocrinology 137, 4817-4826, 1996) and fertilization (See, Amoult, C. et al., Proc. Natl. Acad. Sci. 93, 13004-13009, 1996).

[0004] T-type calcium channel that is quickly activated and inactivated due to a unique low conductivity typically becomes activated between the range of −40 to −30 mV. However, it is very crucial to maintain the cell membrane voltage prior to activation as it may lead to an undesired effect of rapid inactivation. Because the membrane voltage of most cells expressing the T-type calcium channel is not hyperpolarized sufficiently for activating the same, no methods for studying the membrane voltage currently exist with the exception of an electrophysiological method.

[0005] Therefore, studies on signaling pathway mechanism of T-type calcium channel in the nerve cell and scientific researches for developing T-type calcium channel inhibitors are severely undermined. In this respect, it is technically unfeasible, if not impossible, to study and research the T-type calcium channels without resorting to the traditional electrophysiological method. As such, new and innovative methods are needed to improve and enhance the study of the T-type calcium channels.

SUMMARY OF THE INVENTION

[0006] The object of the present invention is to create a certain cell line that can be utilized in scientific studies and/or researches on developing T-type calcium channel inhibitors, and further in screening such inhibitors with a high level of efficiency by activating the T-type calcium channel through changing the extracellular KCl concentration.

[0007] In the cell line of the present invention, α_{1G} T-type calcium channel becomes activated by a high concentration of KCl through expressing the potassium channel. This greatly functions to form a membrane voltage in the human embryonic kidney (HEK293) cell line that expresses the α_{1G} T-type calcium channel stably and consistently so as to lower the membrane voltage toward hyperpolarization and to further stabilize the membrane voltage.

[0008] Thus, the cell line of the present invention is prepared by transforming the HEK293 cell with the human potassium inwardly-rectifying channel (Kir2.1, Genebank accession: AF 153820) gene using a retrovirus expression system. However, it is imperative and thus should be noted herein that the HEK293 cell expresses α_{1G} T-type calcium channel prior to its transformation with the human Kir2.1 gene.

[0009] More specifically, the present invention is directed to a cell line that is prepared by transforming HEK293 cell, stably expressing α_{1G} T-type calcium channel, with a plasmid containing the human Kir2.1 gene. Preferably, the HEK293 cell is transformed with a plamid shown and represented in **FIG. 2** using a retrovirus expression system

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] **FIG. 1** shows a base sequence of the human Kir2.1 gene and an amino acid sequence corresponding thereto.

[0011] **FIG. 2** shows a plasmid pMSCVpuro containing the whole human Kir2.1 gene.

[0012] **FIG. 3** is a graph showing the membrane voltage of HEK293 cell selectively expressing only α_{1G} T-type calcium channel (α_{1G} HEK293 cell, control group) and HEK293 cell co-expressing α_{1G} T-type calcium channel and Kir2.1 (Kir2.1+α_{1G} HEK293 cell);

[0013] **FIGS. 4*a*-4*f*** show the results confirming Kir2.1 expression in Kir2.1+α_{1G} HEK293 cell by using an electrophysiological whole-cell patch-clamp method. **FIG. 4*a*** and **FIG. 4*b*** are graphs illustrating the current-voltage correlation between α_{1G} HEK293 cell and Kir2.1+α_{1G} HEK293 cell. **FIG. 4*c*** and **FIG. 4*d*** show practical current curves of α_{1G} HEK293 cell and Kir2.1+α_{1G} HEK293 cell at each voltage. **FIG. 4*e*** and **FIG. 4*f*** are graphs showing expression of Kir2.1 in Kir2.1+α_{1G} HEK293 cell by an inhibitive level on Ba^{2+} and Cs^{+} known as Kir2.1 inhibitor.

[0014] **FIGS. 5*a*-5*h*** show that Kir2.1 expression does not change the pharmacological properties of α_{1G} T-type calcium channel. **FIG. 5*a*** and **FIG. 5*b*** are graphs illustrating the current-voltage correlation between α_{1G} HEK293 cell and Kir2.1+α_{1G} HEK293 cell for α_{1G} T-type calcium channel activity. **FIG. 5*c*** and **FIG. 5*d*** show practical current curves of α_{1G} HEK293 cell and Kir2.1+α_{1G} HEK293 cell at each voltage. **FIG. 5*e*** and **FIG. 5*f*** are graphs illustrating the current versus concentration of Ni^{2+} known as α_{1G} T-type

calcium channel inhibitor of α_{1G} HEK293 cell and Kir2.1+α_{1G} HEK293 cell. **FIG. 5***g* and **FIG. 5***h* are graphs showing the current versus mibefradil concentration of α_{1G} HEK293 cell and Kir2.1+α_{1G} HEK293 cell.

[0015] **FIGS. 6***a*-**6***b* show that the biophysiological properties of α_{1G} T-type calcium channel are not changed by Kir2.1 expression. **FIG. 6***a* and **FIG. 6***b* are graphs showing the activation/inactivation of T-type calcium channel gate of α_{1G} HEK293 cell and Kir2.1+α_{1G} HEK293 cell.

[0016] **FIG. 7***a* is a graph showing that calcium influx is not changed by the treatment with 70 mM KCl in α_{1G} HEK293 cell; and **FIG. 7***b* is a graph showing that calcium influx is induced by T-type calcium channel activation which is caused by the treatment with 70 mM KCl in Kir2.1+α_{1G} HEK293 cell.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

[0017] The present invention is directed to a cell line that is prepared by transforming HEK293 cell with a human Kir2.1 gene using a retrovirus expression system. It should be specifically noted herein that HEK293 cell expresses α_{1G} T-type calcium channel stably and consistently prior to its transformation with the human Kir2.1 gene.

[0018] The cell line of the present invention has a T-type calcium channel which becomes activated by treatment with a high concentration of KCl. The present cell line was deposited in the Korean Collection for Type Cultures on Sep. 29, 2003 and was assigned accession No. KCTC 10519BP.

[0019] The cell line of the present invention was first established by using HEK293 cell line which expresses α_{1G} T-type calcium channel stably and steadily. It is recognized that HEK293 cell line having such stable α_{1G} T-type calcium channel was originated from Edward Perez-Reyes of University of Virginia (Lee, J. H. et al., J. Neurosci. 19, 1912-1921, 1999).

[0020] In the preferred embodiment of the present invention, the human Kir2.1 gene (SEQ. ID. NO. 1) was treated with the restriction enzymes XhoI and EcoRI. The human Kir2.1 gene was then introduced into pMSCVpuro which was treated with the same enzymes to prepare plasmid Kir2.1-pMSCVpuro. The obtained plasmid was used to transfect a wild type of HEK293 cell and the transfected cells were cultured. The culture was mixed with a culture supernatant of the HEK293 cell line which expressed α_{1G} T-type calcium channel stably and incubated to obtain the cell line of the present invention expressing stable Kir2.1.

[0021] To determine whether the cell line of the present invention forms a stable membrane voltage by Kir2.1 expression, an electrophysiological perforated patch-clamp method was used to determine the cell membrane voltage and the biophysical and pharmacological properties of the expressed Kir2.1. The previously expressed α_{1G} T-type calcium channel were confirmed by whole cell patch-clamp method. Finally, to identify that T-type calcium channel can become activated merely by treatment with a high concentration KCl, change of intracellular calcium ion concentration was determined by using fura-2 AM dye.

[0022] The cell line of the present invention generates an appropriate level of membrane voltage capable of responding to KCl sensitively due to Kir2.1 expression and thus only T-type calcium channel is activated. Therefore, the present cell line can provide the basis for investigating T-type calcium channel in a different way from the traditional electrophysiological method.

[0023] The present invention is further described with the following examples which should not be construed as limiting the present invention. It should be recognized herein that additional modifications and improvements within the spirit and scope of the present invention may be apparent and contemplated.

EXAMPLE 1

Cell Line and Culture Method

[0024] The cell line of the present invention was first created by using HEK293 cell line which already expresses α_{1G} T-type calcium channel stably and consistently. As briefly mentioned above, such cell line had been established by and originated from Edward Perez-Reyes of University of Virginia. The experiments were carried out by using the cell line (α_{1G} HEK293) expressing stable α_{1G} T-type calcium channel as a control and the HEK293 cell line of the present invention (Kir2.1+α_{1G} HEK293) expressing stable Kir2.1 with α_{1G} T-type calcium channel.

[0025] The culture medium of the control was prepared by adding 10% fetal bovine serum and 1% penicillin/streptomycin (v/v) to Dulbecco's modified Eagle's medium (DMEM), and the cell was incubated in a vessel under a humidified condition of 95% air/5% CO_2 at or about 37° C. The culture medium was exchanged with a fresh medium once in 3-4 days and the cell was sub-cultured each week. A solution of geneticin selective antibiotic, G-418 (0.5 mg/ml), was used to grow only the cell which expressed α_{1G} T-type calcium channel.

[0026] The culture medium of Kir2.1+α_{1G} HEK293 cell line was prepard by further adding puromycin (1 μg/ml) to the medium of the control and the culture condition was the same as the control. The cells used in determining T-type calcium channel activity were recorded 2-7 days after culturing the cells on a cover slip coated with poly-L-lysine (0.5 mg/ml) in each sub-culturing.

EXAMPLE 2

Retrovirus Expression System

[0027] The whole base sequence of human Kir2.1 gene shown in **FIG. 1** (SEQ. ID. NO. 1) was cloned into pMSCVpuro (Clontech, **FIG. 2**).

[0028] To obtain a base sequence of Kir2.1, the plasmid cDNA library (Takara) was subjected to PCR with the cycle profiles: 1 cycle of about 5 min at approximately 95° C.; 30 cycles of about 30 sec at approximately 95° C., about 30 sec at approximately 55° C. and about 2 min at approximately 72° C.; and 1 cycle of about 7 min at approximately 72° C. by using Kir2.1 XhoI Forward primer (SEQ. ID. NO. 3) and Kir2.1 RI Reverse primer (SEQ. ID. NO. 4).

(SEQ. ID. NO. 3)
Kir2.1 XhoI Forward primer;
5'-ccgctcgaggccgccatgggcagtgtgag-3'

(SEQ. ID. NO. 4)
Kir2.1 RI Reverse primer;
5'-ccggaattctcatatctccgattctcgcc-3'

[0029] The obtained Kir2.1 gene was treated with XhoI and EcoRI restriction enzymes. The Kir2.1 gene was then introduced into pMSCVpuro which was treated with the same enzymes to prepare plasmid Kir2.1-pMSCVpuro.

[0030] The obtained plasmid was used to prepare the HEK293 cell line in which Kir2.1 was expressed stably as described below.

[0031] The wild type HEK293 cells of about 2.6×10^6 were plated on 6 cm culture dish and grown in a chamber in which DMEM (supplemented with 0.1 mM non-essential amino acids and 10% FBS, culture condition; 37° C., a humidified mixture of 95%:5% (v/v) air and CO_2) was filled. After about 18 hours, the cell line was transfected with a solution that was obtained by precipitating Kir2.1-pMSCVpuro (**FIG. 2**) 6 μg, pEQPAM3 (Clontech) 3 μg and pVSV-G (Clontech) 3 μg in 0.5 M of aqueous calcium solution, and the medium was exchanged with a fresh medium after about 6 hours. After about 20 hours incubation, the medium was exchanged with 3 ml of the medium. After about 20 hours, the culture of the wild type HEK293 cell transfected with such Kir2.1-pMSCVpuro was centrifuged at about 2,000 rpm for approximately 10 minutes. The supernatant of 2.5 ml was mixed with polybrene (Sigma) in a final concentration of 6 mg/ml.

[0032] Separately, the HEK293 cell line of about 2×10^5 and expressing stable α_{1G} T-type calcium channel were plated on a 6 cm culture dish and grown for about 20 hours. Then, the medium was removed and the mixture of the supernatant of the transfected HEK293 cell culture with polybrene, as prepared above, was introduced. After about 6 hours of incubation, the medium was exchanged with fresh medium, and after about further 24 hours of incubation, the medium was again exchanged. After about 24 hours of incubation, 1 μg/ml of puromycin (Sigma) was added to the medium to selectively culture the cells having the puromycin resistance. After culturing the cells selectively during 14 days, the cell line expressing stable Kir2.1 could be obtained. Since the above used cells were selected with 1 mg/ml of G418, 1 mg/ml of G418 was added to the medium when culturing the cells.

EXAMPLE 3

Cell Membrane Voltage Determination

[0033] EPC-9 amplifier (HEKA, Germany) was used to determine a cell membrane voltage difference after expression of inwardly rectifying potassium (IRK)-type potassium channel, Kir2.1, in a single cell level by electrophysiological nystatin-perforated patch-clamp method. An extracellular solution for determining IRK-type potassium channel activity comprised KCl 10 mM, KOH 90 mM, L-aspartic acid 90 mM, $MgCl_2$ 1 mM, NaCl 1 mM and HEPES 10 mM (pH 7.4). An intracellular solution comprised NaCl 140 mM, KCl 3 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1.5 mM, glucose 10 mM and HEPES 10 mM (pH 7.4).

[0034] For determining membrane voltage, the prepared intracellular solution was mixed with nystatin (250 μg/ml) and the resulting solution was introduced into a microglass electrode of 3-4 MΩ resistance. Then, the single cell was pricked with the electrode to determine the cell membrane voltage in a whole-cell recording mode.

[0035] **FIG. 3** is a graph showing the membrane voltage of HEK293 cell wherein only α_{1G} T-type calcium channel is selectively expressed (α_{1G} HEK293 cell, control group), and HEK293 cell wherein α_{1G} T-type calcium channel and Kir 2.1 are co-expressed (Kir2.1+α_{1G} HEK293 cell). According to **FIG. 3**, the membrane voltage of α_{1G} HEK293 cell prior to the expression of Kir2.1 was about −12.2±2.8 mV (n=12). However, the membrane voltage of Kir2.1+α_{1G} HEK293 cell wherein Kir2.1 was expressed was about −57.3±3.7 mV (n=16). This indicated that the stable membrane voltage was formed by the Kir2.1 expression.

EXAMPLE 4

Determination of T-Type Calcium Channel Activity and Kir2.1 Channel Activity

[0036] EPC-9 amplifier (HEKA, Germany) was used to determine the current of T-type calcium channel in a single cell level by electrophysiological whole-cell patch-clamp method. An extracellular solution for determining T-type calcium channel activity comprised NaCl 140 mM, $CaCl_2$ 2 mM and HEPES 10 mM (pH 7.4), whereas an intracellular solution comprised KCl 130 mM, HEPES 10 mM, EGTA 11 mM and MgATP 5 mM (pH 7.4).

[0037] The prepared intracellular solution was introduced into a microglass electrode of about 3-4 MΩ resistance. Then, the single cell was pricked with the electrode to fix the cell membrane voltage as about −100 mV in a whole-cell recording mode. Thereafter, the inward current was determined, in which the current was induced by T-type calcium channel activity when the hypopolarization was caused by about −30 mV (50 ms duration) approximately every 10 seconds.

[0038] Further, the current of IRK-type potassium channel, Kir2.1 was also determined in a single cell level by the whole-cell patch-clamp method. An extracellular solution for determining IRK-type potassium channel activity comprised NaCl 135 mM, KCl 5.4 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1 mM, HEPES 5 mM and glucose 10 mM (pH 7.4). An intracellular solution comprised D-gluconic acid (potassium salt form) 140 mM, $MgCl_2$ 2 mM, EGTA 1 mM, HEPES 5 mM and Na_2ATP 1 mM (pH 7.4).

[0039] After the cell membrane voltage was fixed as about −60 mV in a whole-cell recording mode, −140 to −60 mV of 200 ms step pulse was applied to determine the inward current induced by potassium inwardly-rectifying channel activation of HEK293 cell.

[0040] **FIG. 4** shows the results confiriming Kir2.1 expression in Kir2.1+α_{1G} HEK293 cell by using the electrophysiological whole-cell patch-clamp method. **FIG. 4***a* (α_{1G} HEK293 cell) and **FIG. 4***b* (Kir2.1+α_{1G} HEK293 cell) are graphs showing current-voltage correlation. **FIG. 4***c* (α_{1G} HEK293 cell) and **FIG. 4***d* (Kir2.1+α_{1G} HEK293 cell) show practical current curves at each voltage. When −140 to −60 mV of step pulse was applied, the potassium current was almost not indicated in α_{1G} HEK293 cell (**FIG. 4***c*). Meanwhile, the potassium current was greatly indicated in Kir2.1+α_{1G} HEK293 cell due to the Kir2.1 expression (**FIG. 4***d*). In case that the current activated at each voltage was re-plotted as current-voltage correlation graph, Kir2.1+α_{1G} HEK293 cell (**FIG. 4***b*) showed again the inwardly-rectifying form, thus confirming the Kir2.1 expression.

[0041] Furthermore, the characteristics of Kir2.1 channel were analyzed by comparing with IC_{50} value for Ba^{2+} and Cs^{+}, the conventionally known IRK-type potassium channel inhibitor. For this, Kir2.1 channel activity determination method, as discussed above, was used and the inhibition by the known Kir2.1 inhibitor, Ba^{2+} and Cs^{+}, was observed.

[0042] **FIG. 4***e* and **FIG. 4***f* are graphs showing the maximum current that occurred at each voltage when 1-100 μM Ba^{2+} and 5-500 μM Cs^{+} known as Kir2.1 inhibitor were administered to Kir2.1+α_{1G} HEK293 cell under −140 to −60 mV step pulse. It was re-confirmed that the Kir2.1 expression in Kir2.1+α_{1G} HEK293 cell was inhibited in a concentration-dependent manner by Ba^{2+} and Cs^{+} known as Kir2.1 inhibitor.

[0043] **FIG. 5** shows that Kir2.1 expression does not change the pharmacological properties of α_{1G} T-type calcium channel. **FIG. 5***a* (α_{1G} HEK293 cell) and **FIG. 5***b* (Kir2.1+α_{1G} HEK293 cell) are graphs showing current-voltage correlation for α_{1G} T-type calcium channel activity. **FIG. 5***c* (α_{1G} HEK293 cell) and **FIG. 5***d* (Kir2.1+α_{1G} HEK293 cell) show practical current curves at each voltage, indicating that the current-voltage correlation for α_{1G} T-type calcium channel activity and the magnitude thereof were not changed by the Kir2.1 expression.

[0044] Furthermore, the characteristics of the channel were analyzed by comparing with IC_{50} value for Ni^{2+} and mibefradil, the conventionally known T-type calcium channel inhibitor. About 1 μM to 1 mM of Ni^{2+} and about 0.1 to 10 μM of mibefradil were administered to the cell, respectively, according to the electrophysiological whole-cell patch-clamp method as described above, and then the inhibition (%) of the maximum of the inward current, caused through T-type calcium channel activated at or about −30 mV, was determined.

[0045] **FIG. 5***e* (α_{1G} HEK293 cell) and **FIG. 5***f* (Kir2.1+α_{1G} HEK293 cell) are graphs showing the inhibition (%) of the inward current depending on the concentration of Ni^{2+} known as α_{1G} T-type calcium channel inhibitor. **FIG. 5***g* (α_{1G} HEK293 cell) and **FIG. 5***h* (Kir2.1+α_{1G} HEK293 cell) are graphs showing the current that is dependent on the mibefradil concentration. According to **FIGS. 5***e* to **5***h*, Kir2.1+α_{1G} HEK293 cell is shown to have IC_{50} values for Ni^{2+} and mibefradil that are not significantly different from the values in α_{1G} HEK293 cell. This can be seen that the cell has the pharmacological properties of α_{1G} T-type calcium channel unchanged by Kir2.1 expression.

[0046] **FIG. 6** shows that the biophysiological properties of α_{1G} T-type calcium channel are not changed by Kir2.1 expression. **FIG. 6***a* (α_{1G} HEK293 cell) and **FIG. 6***b* (Kir2.1+α_{1G} HEK293 cell) are graphs showing activation/inactivation of T-type calcium channel gate. The inactivation graph (h_{∞}) was obtained by normalizing maximum inward current obtained under 100 ms step pulse at or about −30 mV after pre-depolarization condition of about −100 to −45 mV at 5 mV interval. The activation graph (m_{∞}) was obtained by normalizing the tail-current obtained by applying −60 to +30 mV of step pulse after fixing the cell membrane valtage as −100 mV. From **FIG. 6**, it can be known that the characteristic activation and inactivation curve of T-type calcium channel are not changed substantially after the Kir2.1 expression.

[0047] **FIG. 7***a* is a graph showing that the treatment with 70 mM KCl does not change calcium influx in the control (α_{1G} HEK293 cell). **FIG. 7***b* is a graph showing that calcium influx is induced by T-type calcium channel activated by treatment with 70 mM KCl in Kir2.1+α_{1G} HEK293 cell.

[0048] The calcium concentration in the cell was determined by using fura-2/AM as a fluorescent Ca^{2+} label. The cells were primarily incubated with 5 μM fura-2/AM and 0.001% Pluronic F-127 in HEPES buffer solution (150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose, pH 7.4) at a room temperature for about 40 to 60 minutes and then washed several times with HEPES buffer solution. After stablilzing the cells for about 10 minutes, the cells were selectively exposed to 340 nm and 380 nm by using an inverted microscope. Thereafter, the emitter fluorescence light that was entered through 515 nm long-pass filter was passed through a cooled CCD carmera. The light was converted into the intracellular calcium concentration by digital fluorescence analyzer to determine the calcium concentration.

[0049] Referring now to **FIG. 7**, it can be seen that the stable membrane voltage was formed by Kir2.1 expression in Kir2.1+α_{1G} HEK293 cell so that calcium influx could be induced by T-type calcium channel activated by treatment of a high concentration of KCl, and not by any electrophysiological method.

[0050] As seen above, since the cell line of the present invention responds sensitively to KCl and forms an appropriate level of the membrane voltage by which only T-type calcium channel is activated, the cell signaling pathway may be investigated by the molecular biological and biochemical studies. Furthermore, since mibefradil that was developed as T-type calcium channel inhibitor cannot be used clinically due to the side effects, the present invention is expected to facilitate T-type calcium channel inhibitor development by using the cell line in screening of lead compound for devoping a new drug, especially in high throughput screening (HTS) technologies.

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